

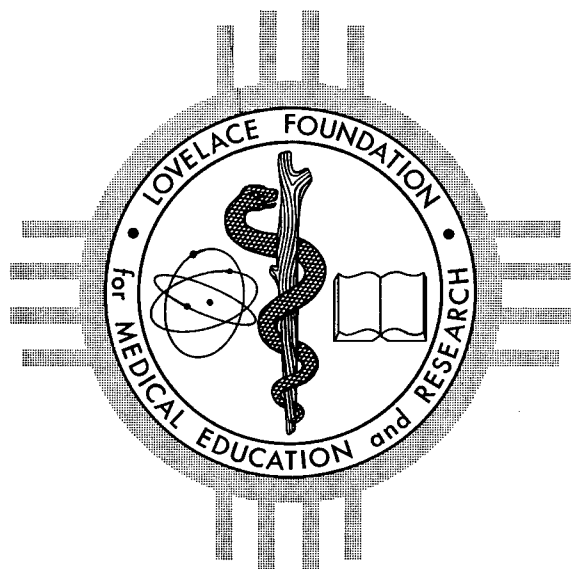
*G. Bowen*  
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Albuquerque, New Mexico

ULTRAMICRO METHODS IN BIOCHEMISTRY  
V. A COMPARISON OF THE ULTRAMICRO  
DETERMINATION OF PHOSPHORUS USING  
N-PHENYL-p-PHENYLENEDIAMINE  
OR 1-AMINO-2-NAPHTHOL-4-SULFONIC ACID  
VI. THE DETERMINATION OF SERUM ALKALINE  
PHOSPHATASE

by

E. VAN STEWART  
AND  
BERNARD B. LONGWELL

December 1964

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PHOSPHORUS USING N-PHENYL-p-PHENYLENEDIAMINE OR  
1-AMINO-2-NAPHTHOL-4-SULFONIC ACID
- VI. THE DETERMINATION OF SERUM ALKALINE PHOSPHATASE

by

E. Van Stewart and Bernard B. Longwell

with the technical assistance of Agnes Wood and Tom Tenney

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## ABSTRACT

An ultramicro method has been developed for the determination of serum inorganic phosphorus which uses 1-amino-2-naphthol-4-sulfonic acid as the reducing agent. This procedure is compared to an ultramicro method which applies the reaction induced by N-phenyl-p-phenylene-diamine. The acceptable wavelengths for the measurement of absorbance in both methods of analyses have been investigated and are discussed. The two methods are comparable in reproducibility and give results which justify the use of either one for the determination of phosphorus.

The method using 1-amino-2-naphthol-4-sulfonic acid has been applied to the determination of serum alkaline phosphatase with a substrate consisting of  $\beta$ -glycerophosphate in a barbiturate buffer at pH 9.3. The ultramicro method is compared to the macro method from which it was derived. The comparisons of results with the two procedures are within acceptable limits to justify recommendation of the ultramicro method for the determination of alkaline phosphatase.

## ACKNOWLEDGMENTS

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# TABLE OF CONTENTS

|   | <u>Page</u> |
|---|-------------|
| ABSTRACT . . . . .  | 1           |
| ACKNOWLEDGMENTS . . . . .   | ii          |
| LIST OF TABLES. . . . .   | iv          |
| LIST OF FIGURES . . . . .   | iv          |
| V. A COMPARISON OF THE ULTRAMICRO DETERMINATION<br>OF PHOSPHORUS USING N-PHENYL-p-PHENYLENEDIAMINE<br>OR 1-AMINO-2-NAPHTHOL-4-SULFONIC ACID . . . . . | 1           |
| INTRODUCTION. . . . .   | 1           |
| METHOD . . . . .  | 1           |
| A. Equipment. . . . .   | 1           |
| B. Reagents Using Aminonaphtholsulfonic Acid (ANSA) . .   | 2           |
| C. Procedure. . . . .   | 2           |
| D. Calculation . . . . .  | 3           |
| E. Method Using N-Phenyl-p-Phenylenediamine (NPPD) .  | 3           |
| RESULTS . . . . .   | 3           |
| A. Choice of Wavelength . . . . .   | 3           |
| B. Standard Curve. . . . .  | 4           |
| C. Standard Deviations . . . . .  | 8           |
| D. Recovery of Phosphorus . . . . .   | 8           |
| E. Comparison of Results . . . . .  | 8           |
| DISCUSSION . . . . .  | 10          |
| SUMMARY . . . . .   | 11          |
| REFERENCES . . . . .  | 11          |
| VI. THE DETERMINATION OF SERUM ALKALINE PHOSPHATASE   | 12          |
| INTRODUCTION. . . . .   | 12          |
| METHOD . . . . .  | 12          |
| A. Reagents . . . . .   | 12          |
| B. Procedure. . . . .   | 13          |
| C. Calculation . . . . .  | 14          |
| RESULTS . . . . .   | 14          |
| A. Linearity of the Reaction . . . . .  | 14          |
| B. Comparison of Ultramicro and Macro Procedures . .  | 14          |
| C. Standard Deviations . . . . .  | 17          |
| SUMMARY . . . . .   | 17          |
| REFERENCES . . . . .  | 18          |

## LIST OF TABLES

|   | <u>Page</u> |
|---|-------------|
| V. A COMPARISON OF THE ULTRAMICRO DETERMINATION<br>OF PHOSPHORUS USING N-PHENYL-p-PHENYLENEDIAMINE<br>OR 1-AMINO-2-NAPHTHOL-4-SULFONIC ACID |             |
| TABLE 1. Comparison of the ANSA and NPPD Methods<br>on Serum Samples . . . . .  | 9           |
| VI. THE DETERMINATION OF SERUM ALKALINE PHOSPHATASE   |             |
| TABLE 1. Comparison of the Macro and Ultramicro<br>Procedures . . . . .   | 16          |

## LIST OF FIGURES

|   | <u>Page</u> |
|---|-------------|
| V. A COMPARISON OF THE ULTRAMICRO DETERMINATION<br>OF PHOSPHORUS USING N-PHENYL-p-PHENYLENEDIAMINE<br>OR 1-AMINO-2-NAPHTHOL-4-SULFONIC ACID                             |             |
| FIGURE 1. Absorbance with ultramicro phosphorus method<br>using 1-amino-2-naphthol-4-sulfonic acid as the<br>reducing agent. . . . .                                    | 5           |
| FIGURE 2. Absorbance values obtained with the ultramicro<br>method for phosphorus analysis which uses<br>N-phenyl-p-phenylenediamine as the reducing<br>agent . . . . . | 6           |
| FIGURE 3. Standardization curves for two ultramicro<br>methods for phosphorus determination . . . . .   | 7           |
| VI. THE DETERMINATION OF SERUM ALKALINE PHOSPHATASE   |             |
| FIGURE 1. Absorbance values which show linearity of the<br>ultramicro alkaline phosphatase procedure<br>described in the text. . . . .                                  | 15          |

## ULTRAMICRO METHODS IN BIOCHEMISTRY

### V. A COMPARISON OF THE ULTRAMICRO DETERMINATION OF PHOSPHORUS USING N-PHENYL-p-PHENYLENEDIAMINE OR 1-AMINO-2-NAPHTHOL-4-SULFONIC ACID

#### INTRODUCTION

The method of Fiske and Subbarow (1) is widely used in clinical chemistry for the determination of phosphorus. Micro modifications of this method, which uses 1-amino-2-naphthol-4-sulfonic acid (ANSA) as the reducing agent, have been fraught with difficulties and it has even been suggested that ANSA could not be used on the ultramicro scale (2, 3). The following report describes an ultramicro procedure using ANSA as the reducing agent. The method is a modification of the method of Kaser and Baker (4). Results obtained with this method are compared to results obtained with the procedure of Dryer, Tammes, and Routh (2) adapted for ultramicro analysis (3). The latter method uses N-phenyl-p-phenylenediamine (NPPD) as the reducing agent.

#### METHOD

##### A. Equipment

The main components of the Sanz-Spinco ultramicro clinical chemistry system (5) were used with the following exceptions:

1. Pipettes. All pipettes larger than 20  $\mu$ l capacity were made and calibrated in this laboratory.
2. Colorimeter. Both the Coleman Junior spectrophotometer equipped with an ultramicro cell and the Spinco spectrophotometer which had been modified to accept the Coleman ultramicro cell were used.
3. Mixer. A Vortex Junior Mixer (Scientific Industries, Queens Village, New York) was substituted for the Spinco micromixer.



## B. Reagents for Using Aminonaphtholsulfonic Acid (ANSA)

1. Phosphorus standard (stock) (40 mg/100 ml). Dissolve 0.176 gm of dried potassium dihydrogen phosphate ( $\text{KH}_2\text{PO}_4$ ) in distilled water and dilute to 100 ml. The stability is increased if this standard is kept in the refrigerator.

2. Dilute standard (4 mg/100 ml). Dilute 1.0 ml of the stock standard to 10 ml with water.

3. Trichloroacetic acid (10%). Dissolve 10 gm of trichloroacetic acid in distilled water and make up to 100 ml.

4. Molybdic acid. Dissolve 25 gm of reagent grade ammonium molybdate in 500 ml of distilled water. Add 300 ml of 10 percent sulfuric acid and dilute to one liter with water.

5. Aminonaphtholsulfonic acid (ANSA). Dissolve 14.6 gm of sodium hydrogen sulfite ( $\text{NaHSO}_3$ ) and 0.5 gm of sodium sulfite ( $\text{Na}_2\text{SO}_3$ ) in distilled water and make up to 100 ml. Add 0.1 gm of powdered 1-amino-2-naphthol-4-sulfonic acid and shake until it has dissolved. Store in a brown bottle in the refrigerator. If the solution is colored, filter through activated charcoal.

## C. Procedure

1. To a 400  $\mu\text{l}$  polyethylene test tube add 10  $\mu\text{l}$  of blank (distilled water), standard, or serum followed by 150  $\mu\text{l}$  of 10 percent trichloroacetic acid.

2. Mix thoroughly and let stand for two minutes, then centrifuge for one and one-half minutes.

3. Transfer a 100  $\mu\text{l}$  aliquot to a clean micro test tube.

4. Add 20  $\mu\text{l}$  of molybdic acid and mix. Then add 20  $\mu\text{l}$  of ANSA and mix thoroughly.

5. Let stand for 15 minutes and read absorbance against water at 650  $\text{m}\mu$  with the Spinco spectrophotometer, or 680  $\text{m}\mu$  with the Coleman Junior spectrophotometer, in a micro cuvette.

#### D. Calculation

$$\frac{\text{Absorbance (A) of unknown} - \text{A of blank}}{\text{A of standard} - \text{A of blank}} \times 4 = \text{mg phosphorus/100 ml}$$

#### E. Method Using N-Phenyl-p-Phenylenediamine (NPPD)

The method of Dryer, Tammes, and Routh(2) as modified for ultra-micro analysis by Knights, MacDonald, and Ploompue (3) was used as described, with the exception of one experiment in which a 20 $\mu$ l sample was substituted for the 25 $\mu$ l sample originally suggested,

### RESULTS

#### A. Choice of Wavelength

Some instruments do not provide a selection of wavelength greater than 650 m $\mu$ . Because of this limitation, the feasibility of phosphorus analyses with absorbance measurements at wavelengths other than the point of maximum absorptivity was investigated. Absorbances were measured at 650, 680, 700, and 745 m $\mu$  with standards equivalent to 2 to 10 mg phosphorus per 100 ml. The readings were made with a Beckman DU spectrophotometer modified for ultramicro analysis and equipped with the Gilford<sup>1</sup> Instrument multiple sample absorbance recorder.

Curves 1, 2, and 3 (Figure 1) show the results obtained with the ANSA method as described above when the absorbance measurements were made at 700, 680, and 650 m $\mu$ , respectively. Curves 4, 5, and 6 record measurements made at the same wavelengths, but with the method modified to use a 20 $\mu$ l sample with a final dilution of the color reaction to 320 $\mu$ l, the same volume used in the NPPD method. Figure 2 presents standardization data for the NPPD method with a 20 $\mu$ l rather than the 25 $\mu$ l sample originally prescribed. The absorbance measurements were made at 700, 680, and 650 m $\mu$  for Curves 1, 2, and 3, respectively.

---

<sup>1</sup>Gilford Instrument Laboratories, Inc., Oberlin, Ohio.

The graph of the results at 700 and 745  $m\mu$  for both methods was so nearly identical that only the values obtained at 700  $m\mu$  are plotted. The comparative values are shown below.

| Phosphorus<br>concentration<br>mg per 100 ml | ANSA Method |            | NPPD Method |            |
|--|-------------|------------|-------------|------------|
|  | 700 $m\mu$  | 745 $m\mu$ | 700 $m\mu$  | 745 $m\mu$ |
| 2  | 0.086       | 0.083      | 0.063       | 0.063      |
| 4  | 0.175       | 0.172      | 0.125       | 0.126      |
| 6  | 0.265       | 0.264      | 0.188       | 0.190      |
| 10   | 0.441       | 0.439      | 0.322       | 0.324      |

It is evident that wavelengths from 650 to 745  $m\mu$  may be used with either of these methods to give results that are acceptable for the analyses of serum phosphorus. The differences in absorbances are explained by differences in sample size, final color reaction volumes and color intensity developed.

#### B. Standard Curve

The results recorded in Figure 3 show the absorbance values for phosphorus standards of varying concentrations. Curve 1 shows the results with the ANSA method as described herein under Procedure ( $10\mu l$  sample, final color reaction volume  $140\mu l$ ). The absorbance measurements were made at 680  $m\mu$  with the Coleman Junior spectrophotometer equipped with an ultramicro cuvette. Curve 2 records results with the ANSA method modified by using a  $20\mu l$  sample and making a final dilution to  $320\mu l$  to correspond to the final volume of the color reaction of the NPPD method. Curve 3 shows the results of the NPPD method as described by Knights, MacDonald, and Ploompuu (3) with their designated  $25\mu l$  sample. The readings for Curve 2 were made at 680  $m\mu$  and for Curve 3 at 700  $m\mu$ . All absorbance measurements were made with the Coleman Junior spectrophotometer.

Absorbance is linear with concentration by both methods over the ranges recorded in Figure 2, up to 10 mg phosphorus per 100 ml. The close agreement of the results obtained with the ANSA method, modified

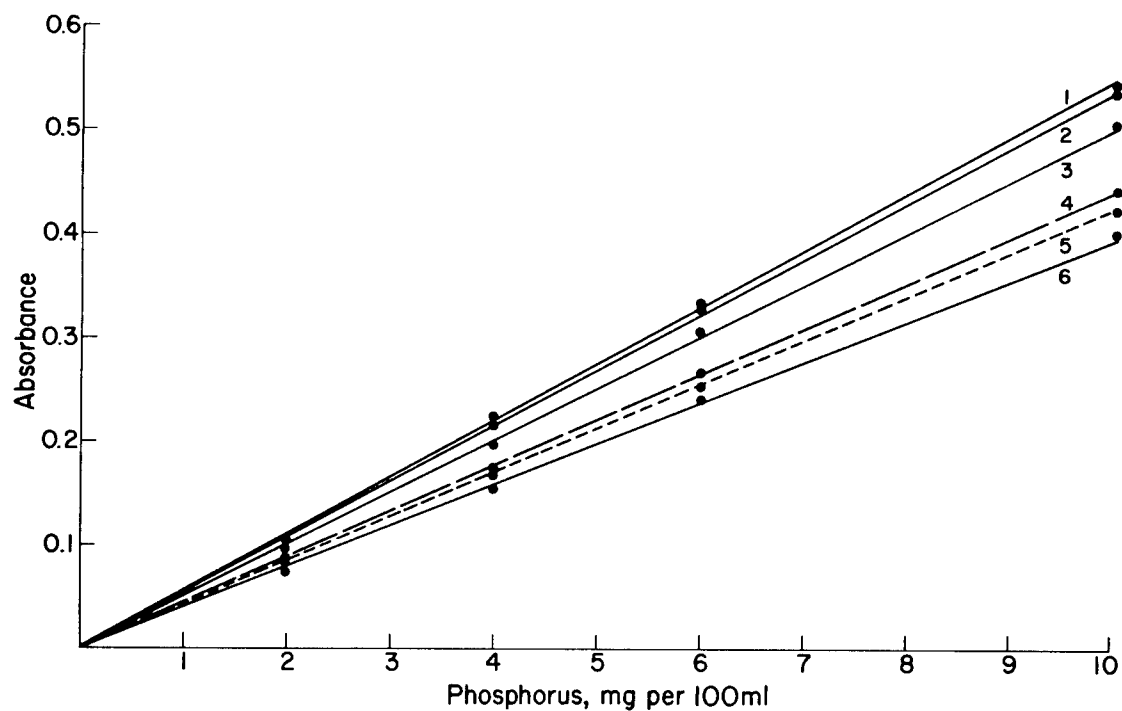


Fig. 1. Absorbance with ultramicro phosphorus method using 1-amino-2-naphthol-4-sulfonic acid as the reducing agent. Curves 1, 2, and 3 show absorbance values at 700, 680, and 650  $m\mu$ , respectively, with the method as described in the text. Curves 4, 5, and 6 show absorbance values at 700, 680, and 650  $m\mu$  obtained with the method modified to use a 20  $\mu$ l sample followed by dilution of the color reaction to 320  $\mu$ l.

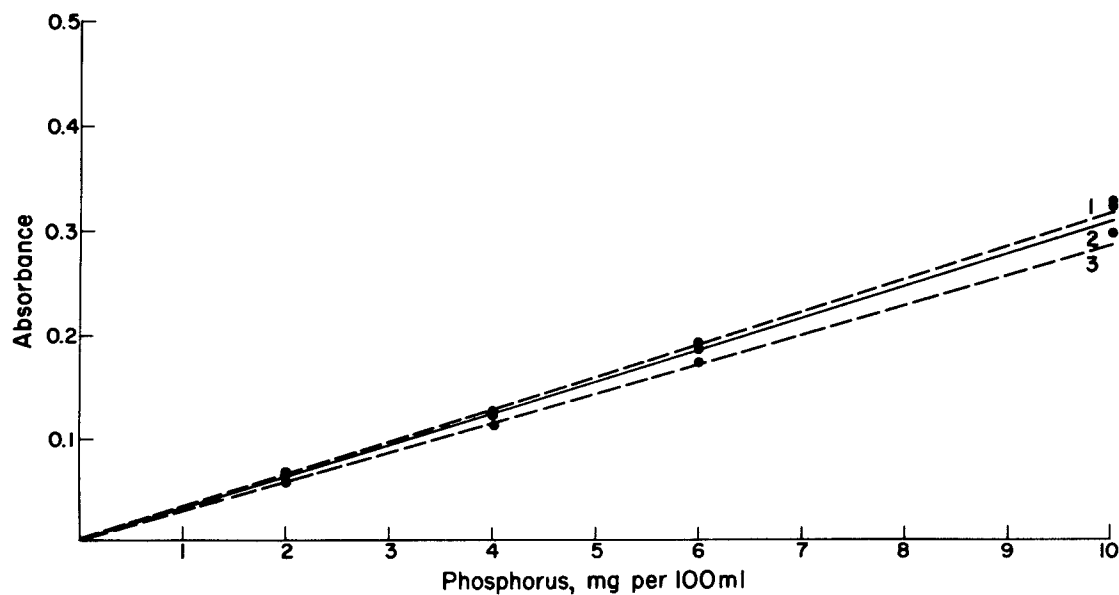


Fig. 2. Absorbance values obtained with the ultramicro method for phosphorus analysis which uses N-phenyl-p-phenylenediamine as the reducing agent (3). The method was modified to use a  $20\mu$ l sample. Curves 1, 2, and 3 were obtained at 700, 680, and 650  $m\mu$ , respectively.

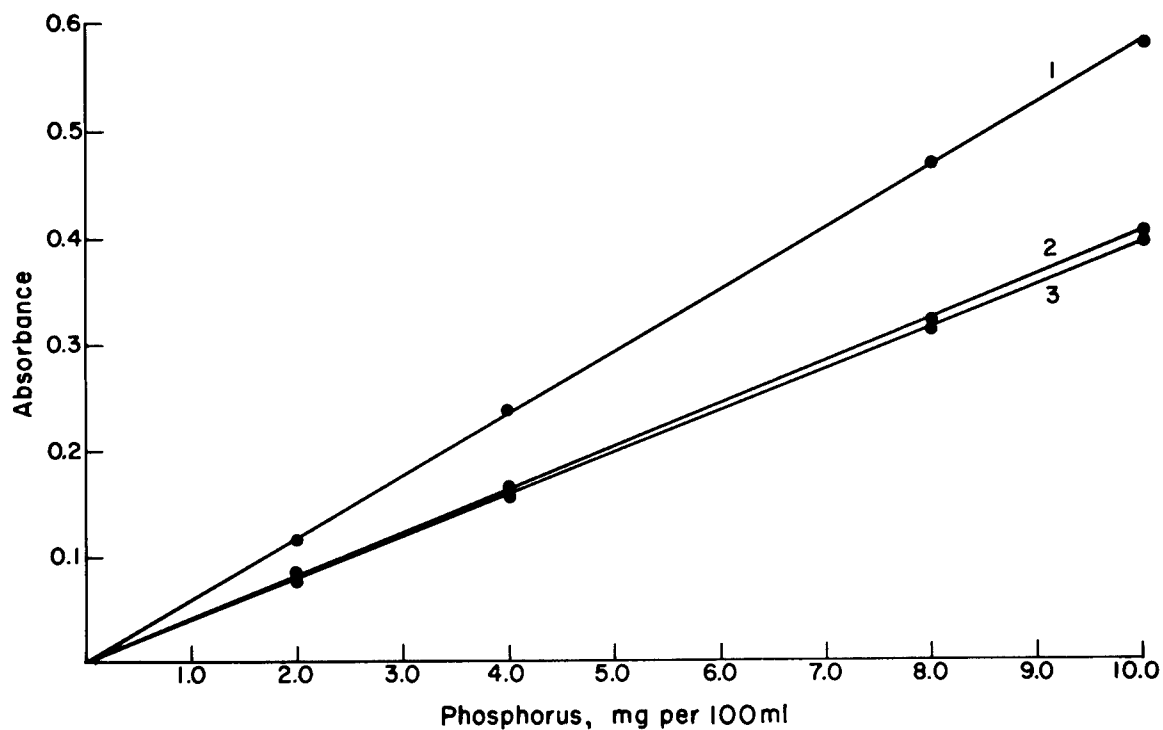


Fig. 3. Standardization curves for two ultramicro methods for phosphorus determination. Curve 1, the method using 1-amino-2-naphthol-4-sulfonic acid described in the text. Curve 2, the aminonaphtholsulfonic acid method modified to use  $20\mu\text{l}$  of sample followed by final color reaction dilution to  $320\mu\text{l}$ . Curve 3, method using N-phenyl-p-phenylenediamine as the reducing agent (3).

to compare approximately in sample size and exactly in final dilution with the NPPD method, indicates that there is little to choose between the two reducing agents applied to the ultramicro determination of phosphorus.

#### C. Standard Deviations

Standard deviations were determined for both procedures by doing thirty analyses on the same samples. A different serum sample was used for the determination of the standard deviation for each of the methods. The modified Fiske and Subbarow method gave a standard deviation of  $\pm 0.05$  on a serum with a mean phosphorus value of 3.2 mg per 100 ml and a standard deviation of  $\pm 0.12$  was obtained on a serum with a mean phosphorus value of 4.46 mg per 100 ml of serum by the NPPD procedure.

#### D. Recovery of Phosphorus

Dried crystalline potassium dihydrogen phosphate was added to an aliquot of a fresh serum sample which was then mixed thoroughly until the crystals were dissolved. The phosphorus was determined on an aliquot without added phosphorus and on an aliquot with added phosphorus by both methods. By calculation, 3.7 mg of phosphorus was added per 100 ml of serum. A recovery of 3.8 mg per 100 ml was obtained with the NPPD method and 3.76 mg per 100 ml with the ANSA procedure, recoveries of 103 and 102 percent, respectively.

#### E. Comparison of Results

Thirty serum samples were analyzed for phosphorus by both methods. A comparison of the results is shown in Table 1. Good agreement was obtained between the methods on the majority of samples. The exceptions were those sera which were lipemic. In the lipemic samples, no definite trend was apparent which indicated that one method was giving consistently higher results than the other. In two of the samples, the value obtained by the ANSA method was higher, while in three of the samples, the NPPD method gave the higher values. No explanation

TABLE 1  
COMPARISON OF THE ANSA AND NPPD METHODS ON SERUM SAMPLES

| Sample | mg of Phosphorus per 100 ml of serum |             |
|--------|--------------------------------------|-------------|
|        | ANSA Method                          | NPPD Method |
| 1      | 3.9                                  | 4.0         |
| 2      | 3.1                                  | 3.3         |
| 3      | 3.9                                  | 4.1         |
| 4      | 4.4                                  | 4.2         |
| 5      | 4.0                                  | 3.9         |
| 6      | 4.4                                  | 4.9 *       |
| 7      | 3.9                                  | 4.4         |
| 8      | 3.3                                  | 3.1         |
| 9      | 3.9                                  | 3.9         |
| 10     | 4.4                                  | 4.4         |
| 11     | 3.9                                  | 4.0         |
| 12     | 5.8                                  | 6.5 *       |
| 13     | 4.7                                  | 4.7         |
| 14     | 3.6                                  | 3.4         |
| 15     | 3.6                                  | 3.3         |
| 16     | 3.6                                  | 3.3         |
| 17     | 3.8                                  | 3.2 *       |
| 18     | 3.7                                  | 3.8         |
| 19     | 4.1                                  | 3.9         |
| 20     | 3.4                                  | 3.4         |
| 21     | 4.1                                  | 4.2         |
| 22     | 3.4                                  | 3.8         |
| 23     | 3.9                                  | 3.8         |
| 24     | 4.2                                  | 3.7 *       |
| 25     | 4.3                                  | 4.8 *       |
| 26     | 3.5                                  | 3.6         |
| 27     | 4.3                                  | 4.1         |
| 28     | 4.0                                  | 3.8         |
| 29     | 12.5                                 | 12.2        |
| 30     | 5.2                                  | 5.0         |

\* Lipemic serum.



of these results, or indication of which, if either, method is more accurate in the presence of lipemia, can be advanced.

### DISCUSSION

The results obtained by both the ultramicro NPPD and the ANSA methods are quite accurate and reproducible. Neither method appeared to have enough advantages over the other that it could be recommended as the method of choice.

The longest wavelength that can be used on the Spinco spectrophotometer is 650  $m\mu$ . Dryer et al. (2) showed that absorbance at 770  $m\mu$  is higher than at 650  $m\mu$ . The question, therefore, arose concerning whether wavelengths other than the maximum previously investigated could be used justifiably. This question seems to be answered by the data presented in Figures 1 and 2 up to a wavelength of 745  $m\mu$ . There is not sufficient difference between the absorbance values at the four wavelengths investigated to invalidate the use of any one of them for the determination of phosphorus. A determination of the ratios of absorbance at 770  $m\mu$  and 650  $m\mu$  for the two methods, using the Beckman DK-1A spectrophotometer, gave 1.19 for the NPPD method and 1.13 for the ANSA method.

During the transfer of the supernatant solution after precipitation and centrifugation of the proteins of some serum samples, a small portion of the precipitate was transferred in both methods. Longer periods of centrifugation did not seem to allow a cleaner transfer because a portion of the precipitate still remained on the surface around the edges of the liquid. It was found, however, that the interference from this precipitate could be eliminated by centrifuging the sample for one minute after the color had been developed for 15 minutes.

## SUMMARY

A method for the ultramicro determination of phosphorus utilizing aminonaphtholsulfonic acid as the reducing agent has been evaluated and is compared to a method which employs N-phenyl-p-phenylenediamine. Either method is satisfactory for this determination.

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ULTRAMICRO METHODS IN BIOCHEMISTRY  
VI. THE DETERMINATION OF SERUM ALKALINE PHOSPHATASE

INTRODUCTION

Since the introduction of the Bodansky method for the determination of serum alkaline phosphatase (1), modifications of this procedure (2, 3) have been described, and several other approaches to the determination of this enzyme have been proposed (4, 5, 6). Procedures for ultramicro or micro analyses of alkaline phosphatase have been described by Bessey, Lowry, and Brock (4) and by O'Brien (7), Knights, MacDonald and Ploompau (8), and Meites (9).

The present report describes the ultramicro determination of alkaline phosphatase by a modification of the method of Kaser and Baker (3) in which the phosphorus determination is accomplished as previously described (10).

METHOD

A. Reagents

1. The stock phosphorus standard, the dilute standard, the molybdic acid reagent and the aminonaphtholsulfonic acid reagent are identical to the same solutions described in the procedure for the determination of inorganic phosphorus (10).

2. Trichloroacetic acid (30%). Dissolve 30 gm of reagent grade trichloroacetic acid in distilled water and dilute to 100 ml.

3. Substrate. Dissolve 1.25 gm of sodium- $\beta$ -glycerophosphate ( $C_3H_9PO_6 \cdot 5H_2O$ ) and 1.06 gm of sodium diethyl barbiturate in approximately 100 ml of distilled water. Make up to 250 ml. Adjust the pH to 9.8 at 25°C, using either sodium hydroxide or hydrochloric acid. Divide the substrate into 10-20 ml portions and freeze. Keep frozen until used.

## B. Procedure

1. To a 400  $\mu$ l polyethylene test tube add 150  $\mu$ l of substrate and 10  $\mu$ l of serum. Mix thoroughly and incubate at 37°C for one hour.

While the phosphatase tubes (Procedure, Paragraph 1) are incubating, prepare the blanks, standard, and serum samples for the determination of inorganic phosphorus as follows:

2. To a 400  $\mu$ l polyethylene test tube add 150  $\mu$ l of substrate and 10  $\mu$ l of blank (distilled water), standard, or serum. Immediately after the addition of a sample to each tube, add 50  $\mu$ l of 30 percent trichloroacetic acid and mix thoroughly.

3. Let the sample stand at room temperature for 15 minutes, then centrifuge for one and one-half minutes.

4. Transfer 100  $\mu$ l of the supernatant solution to a clean 400  $\mu$ l polyethylene test tube. Stopper these tubes and let them stand to be analyzed together with the samples that have been incubated (Procedure, Paragraph 1).

When the prescribed period of incubation of the phosphatase tubes is complete (Procedure, Paragraph 1), proceed as follows:

5. Add 50  $\mu$ l of 30 percent trichloroacetic acid to each of the phosphatase tubes. Mix thoroughly and let stand for 15 minutes at room temperature.

6. Centrifuge these tubes for one and one-half minutes and transfer 100  $\mu$ l of the supernatant solution to clean 400  $\mu$ l polyethylene test tubes.

Then to both the phosphatase tubes and to the tubes prepared for serum inorganic phosphorus analysis, continue as follows:

7. Add 20  $\mu$ l of molybdic acid to the transferred supernatant solution and mix, then add 20  $\mu$ l of ANSA and mix thoroughly. Let the tubes stand for 15 minutes at room temperature then read them against water at 650 m $\mu$ .

The method was developed and subsequent data were collected using the Spinco spectrophotometer, the upper wavelength limit of which is 650 m $\mu$ . Other instruments set at higher wavelengths may be used equally well.

### C. Calculation

$$\frac{\text{Absorbance (A) of unknown} - \text{A of blank}}{\text{A of standard} - \text{A of blank}} \times 4 = \text{concentration of inorganic phosphorus (mg/100 ml)}$$

$$\begin{array}{rcl} \text{Phosphorus determined} & & \text{inorganic} \\ \text{in alkaline phosphatase} & - & \text{phosphorus} \\ \text{(incubated) tubes} & & \text{determined} \end{array} = \begin{array}{r} \text{alkaline} \\ \text{phosphatase} \\ \text{in units} \end{array}$$

Calculate the inorganic phosphorus in the tubes which have been incubated and those which have not in the same manner. A unit of phosphatase activity is defined as the amount of reaction that will release one mg of inorganic phosphorus per 100 ml of serum after one hour incubation at 37°C under the conditions of pH, buffer concentration, and type of substrate herein described.

## RESULTS

### A. Linearity of the Reaction

In order to demonstrate the linearity of the reaction, alkaline phosphatase was obtained as a dry powder from Nutritional Biochemical Corporation and dissolved in 0.9 percent sodium chloride solution. It was then diluted with the sodium chloride solution until the activity could be determined. Aliquots of this solution were then further diluted 1:2, 1:4, and 1:8. The phosphatase activity of each of these dilutions was then determined and expressed in units. The enzyme activity is plotted against the fractional dilution in Figure 1. A linear plot was obtained indicating that the catalytic release of phosphorus is directly related to the activity of the enzyme in the range possible with the procedure.

### B. Comparison of Ultramicro and Macro Procedures

A comparison of the results obtained by a macro procedure (3) with those obtained by the ultramicro procedure using human serum are listed in Table 1. In none of the determinations did the macro and ultramicro results vary as much as one unit. The maximum

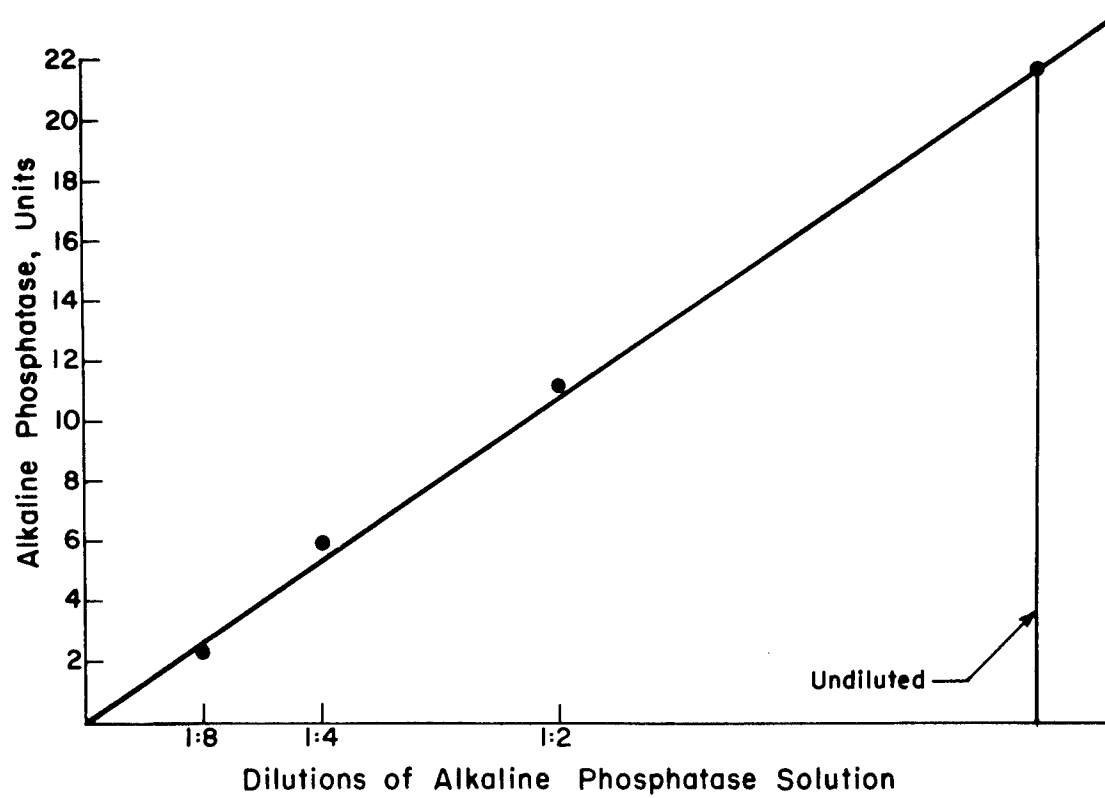


Fig. 1. Absorbance values which show linearity of the ultramicro alkaline phosphatase procedure described in the text.

TABLE 1

## COMPARISON OF THE MACRO AND ULTRAMICRO PROCEDURES

The inorganic phosphorus and alkaline phosphatase were determined on the same sample by the macro and ultramicro procedures.

| Sample | Inorganic Phosphorus<br>mg per 100 ml |            |             | Alkaline Phosphatase<br>Units |            |             |
|--------|---------------------------------------|------------|-------------|-------------------------------|------------|-------------|
|        | Macro                                 | Ultramicro | Difference* | Macro                         | Ultramicro | Difference* |
| 1      | 4.5                                   | 4.9        | +0.4        | 10.3                          | 9.4        | -0.9        |
| 2      | 3.7                                   | 4.0        | +0.3        | 2.1                           | 2.1        | 0.0         |
| 3      | 3.8                                   | 3.7        | -0.1        | 4.4                           | 4.5        | +0.1        |
| 4      | 4.2                                   | 3.9        | -0.3        | 9.3                           | 9.3        | 0.0         |
| 5      | 4.3                                   | 4.4        | +0.1        | 8.1                           | 7.8        | -0.3        |
| 6      | 2.8                                   | 3.1        | +0.3        | 3.0                           | 2.9        | -0.1        |
| 7      | 3.6                                   | 3.8        | +0.2        | 4.2                           | 4.0        | -0.2        |
| 8      | 3.5                                   | 3.6        | +0.1        | 3.1                           | 3.0        | -0.1        |
| 9      | 3.3                                   | 3.6        | +0.3        | 3.9                           | 3.9        | 0.0         |
| 10     | 2.8                                   | 2.9        | -0.1        | 2.8                           | 3.5        | +0.7        |
| 11     | 3.7                                   | 4.0        | +0.3        | 6.0                           | 6.7        | +0.7        |
| 12     | 4.5                                   | 4.4        | -0.1        | 3.1                           | 3.1        | 0.0         |
| 13     | 2.7                                   | 3.0        | +0.3        | 3.9                           | 3.6        | -0.3        |
| 14     | 3.7                                   | 4.2        | +0.5        | 7.5                           | 7.0        | -0.5        |
| 15     | 4.0                                   | 4.2        | +0.2        | 5.4                           | 4.6        | -0.8        |

\* The ultramicro result is compared to the macro result as reference.

variation was 0.9 unit but was 0.5 unit or more in only 5 of the 15 analyses. Variations in the determination of alkaline phosphatase by this method are influenced by variations in the determination of inorganic phosphorus originally present in the serum. The comparative results by the two procedures for inorganic phosphorus are also listed in Table 1. The ultramicro procedure compares favorably with the macro procedure for both inorganic phosphorus and alkaline phosphatase.

#### C. Standard Deviations

Thirty ultramicro alkaline phosphatase determinations were performed on the same sample of human serum. The mean value found was 3.4 units with a standard deviation of  $\pm 0.05$  mg of phosphorus per 100 ml of serum.

### SUMMARY

An ultramicro procedure for the determination of alkaline phosphatase has been investigated. The method utilizes sodium  $\beta$ -glycerophosphate as the substrate at pH 9.3, and the phosphorus is determined by ultramicro analysis of inorganic phosphorus using 1-amino-2-naphthol-4-sulfonic acid as the reducing agent (10). Results with the method are compared to results obtained with the macro method from which the ultramicro method was derived.



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### VI. THE DETERMINATION OF SERUM ALKALINE PHOSPHATASE

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